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A *Listeria monocytogenes* Mutant Defective in Bacteriophage Attachment Is Attenuated in Orally Inoculated Mice and Impaired in Enterocyte Intracellular Growth[▽]

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A *Listeria monocytogenes* bacteriophage was used to identify a phage-resistant Tn*917* insertion mutant of the mouse-virulent listerial strain F6214-1. The mutant was attenuated when it was inoculated orally into female A/J mice and failed to replicate efficiently in cultured mouse enterocytes. Phage binding studies indicated that the mutant had a cell surface alteration that precluded phage attachment. All phenotypes associated with the mutation could be complemented in *trans* by a single open reading frame (ORF) that corresponded to the ORF interrupted by the Tn*917* insertion. The complementation effected was, in all cases, at a level indistinguishable from that of the parent. The Tn*917* insertion interrupted a gene that is predicted to encode a group 2 glycosyl transferase (provisionally designated *glcV*). A similar *glcV* gene is present in *Listeria welshimeri* and *Listeria innocua* and in some serotypes of *L. monocytogenes*. We speculate that the loss of the *glcV* product results in a defective phage receptor and that this alteration coincidentally influences a feature of the normal host-pathogen interaction required for virulence. Interestingly, the *glcV* lesion, while preventing phage attachment, did not alter the mutant's ability to bind to cultured mouse enterocyte monolayers. Rather, the mutation appeared to alter a subsequent step in intracellular replication measured by a reduction in plaque-forming efficiency and plaque size. In vivo, the mutant was undetectable in the liver and spleen 48 h after oral inoculation. The mutation is significant in part because it is one of the few that produce attenuation when the mutant is delivered orally.

Listeria monocytogenes (6) is a gram-positive bacterial pathogen that is the cause of listeriosis in humans and a variety of other animals (16). Characteristic steps leading to listeriosis include (i) ingestion of a contaminated food source, (ii) colonization of the intestinal lumen, (iii) escape from the intestinal lumen, (iv) lodgment and replication in the liver and spleen, and (v) resolution (which is dependent upon T-cell-mediated immunity) or hematogenous spread to other organs. In hosts with a fully functional immune system, the infection is effectively cleared, and the immunity that results is long lived (22). Listeriae are frequent contaminants of foodstuffs (14), and food-borne outbreaks of listeriosis are relatively common (7). When such outbreaks occur, the percentage of fatalities in predisposed individuals (pregnant women, neonates, and immunosuppressed individuals) can exceed that due to other more notorious food-borne infectious and intoxicating agents, such as *Salmonella* sp. and *Clostridium botulinum* (23).

Identification of listerial virulence factors has been an active area of research since the role of *L. monocytogenes* in human food-borne outbreaks was first appreciated (34). The current picture of *L. monocytogenes*-host cell interactions and intracellular growth is one of the most sophisticated known (12). Nevertheless, the steps in the pathogenesis of listeriosis have been defined, to a large extent, by using listerial mutants that fail to overcome a specific host defense (26). Consequently, the identification and characterization of attenuated mutants remain an active endeavor.

Many strategies have been employed to identify virulence factors via genetic means (2, 29, 37). The most recent approaches use genomics, followed by site-directed mutagenesis and testing of the mutants in vitro and in vivo for defects in some aspect of host infectivity or in intracellular growth (3, 4, 33). One approach that has been successful with other pathogenic bacteria has been to use bacteriophage (phage) to obtain mutants with cell surface alterations that preclude phage binding and then to screen the resistant mutants for failure to infect an appropriate host (11, 31, 35, 39). Such mutants are useful because the phage not only can be employed as a selective or screening agent but also can be used subsequently as an analytical tool to identify the role of the phage receptor in host-bacterium interactions at the molecular level (15).

In this study we employed a derivative of *L. monocytogenes* phage P35 (19) to identify a resistant Tn*917* insertion mutant of the mouse-virulent strain F6214-1, and we documented that the mutant failed to bind phage and was attenuated when it was inoculated orally into mice. The mutant also failed to replicate efficiently in cultured mouse enterocytes (MODE K cells), although it bound to these cells with an efficiency indistinguishable from that of the parent. We further showed that all phenotypes associated with the mutation were comple-

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TABLE 1. Bacteria, bacteriophages, and plasmids used in this study

Bacterial strain, phage, or plasmid	Description[a]	Reference or source
Bacterial strains		
F6214-1	*L. monocytogenes* serotype 4nonb, sensitive to phage P35h4, Str^r Nal^r	18
PAS394	F6214-1 (*glcA*::Tn*917*) Str^r Nal^r Lm^r Em^r, phage P35h4 resistant	This study
DH5-α	*E. coli* cloning strain	Invitrogen
Phages		
P35	Parental phage (also called LUMP35); host, *L. monocytogenes* serotype 1/2a strains	19
P35h4	Host range mutant of P35	This study
Plasmids		
pGEM	*E. coli* cloning vector; Ap^r	Promega
pKSV7[b]	*L. monocytogenes*/*E. coli* cloning vector; Ts Cm^r Ap^r	38
pPAS25	pGEM *glcV pmpA*	This study
pPAS29	pKSV7 *glcV pmpA*	This study
pPAS94	pKSV7 *glcV* Δ*pmpA1*	This study
pPAS64	pKSV7 *glcV* Δ*pmpA2*	This study
pPAS97	pKSV7 Δ*glcV1 pmpA*	This study
pPAS65	pKSV7 Δ*glcV2 pmpA*	This study

[a] Abbreviations: Str^r, streptomycin resistant; Nal^r, nalidixic acid resistant; Lm^r, lincomycin resistant; Em^r, erythromycin resistant; Cm^r, chloramphenicol resistant; Ap^r, ampicillin resistant; Ts, temperature-sensitive replication.
[b] Temperature-sensitive replication in *L. monocytogenes* was observed at temperatures above ca. 42°C.

mented in *trans* by the product of a single open reading frame (ORF) (designated *glcV*). The *glcV* gene presumptively encodes a group 2 glycosyl transferase. We think that the loss of this product results in a defective phage receptor and that this defect coincidentally alters some feature of the normal host-pathogen interaction required for virulence. Interestingly, the change in the *L. monocytogenes* cell surface did not alter the ability of the mutant to bind to cultured mouse enterocytes (as one might expect). Rather, the mutation appeared to alter a subsequent step in intracellular replication.

MATERIALS AND METHODS

Bacterial strains, bacteriophage, plasmids, and media. The *L. monocytogenes* strains and bacteriophage derivatives employed in this study are listed in Table 1. Our parental *L. monocytogenes* strain, F6214-1, is a spontaneous streptomycin-resistant derivative of serotype 4nonb strain F6214 (18). Depending on the experimental situation, listerial strains were propagated at temperatures ranging from 22 to 44°C in either L broth (24) or brain heart infusion (BHI) broth or on those media supplemented with 1.5% agar. Our pKSV7 listerial temperature-sensitive cloning vector was examined for stability during growth at temperatures ranging from 31 to 44°C. We observed no increase in plasmid instability at temperatures between 31 and 37°C (this allowed use of the plasmid in enterocyte tissue culture and in vivo). Plasmid loss was measurable at ca. 42°C and at higher temperatures. Broth-grown bacteria destined for mouse inoculation were prepared as described by Hamrick et al. (18). Where indicated, antibiotics were incorporated into the media at the following concentrations: streptomycin, 50 μg/ml; nalidixic acid, 40 μg/ml; erythromycin, 1 μg/ml; lincomycin, 25 μg/ml; chloramphenicol, 10 μg/ml; and ampicillin, 100 μg/ml.

Listerial strains grown for preparation of phage lysates or for phage binding assays were propagated in L broth or on L agar supplemented with 10 mM $CaCl_2$ and 10 mM $MgSO_4$ (referred to as LCM media). Lysate preparation involved removing a portion of an overnight culture of F6214-1 (typically 0.2 ml containing approximately 5×10^8 CFU) and mixing it with an aliquot of a preexisting P35h4 phage lysate in a 15-ml test tube to obtain a ratio of approximately 1 PFU per 10^4 CFU in a total volume of 0.3 ml. After 20 min of absorption at 22°C, equal volumes (3 ml each) of soft agar (LCM broth with 0.7% agar) and plain LCM broth were added. The contents were mixed and poured onto a freshly prepared L agar plate. After incubation for 15 to 24 h at 22°C, lysates were harvested by scraping the soft agar layer into 40-ml centrifuge tubes. The tubes containing the overlay were centrifuged at $7,688 \times g$ for 10 min to remove cell debris and agar. The resulting supernatant was filtered through a 0.45-μm filter, and the sterile lysate was stored at 4°C. Lysates had average titers of 5×10^9 PFU/ml. (Titration was performed essentially as described above for lysate preparation except that 3 ml of soft agar overlay was employed with lysate serial dilutions in 0.15 N NaCl.)

Mutagenesis. Bacteriophage P35h4 was isolated as a host range mutant of LMUP35 (P35) (19) following hydroxylamine mutagenesis (24) and selection for mutants that could form plaques on strain F6214-1. A plaque-purified derivative of one such mutant was designated P35h4. The lesion in P35h4 expands the host range of P35 to include listerial serotypes other than serotype 1/2a, such as our serotype 4nonb strain (19). An *L. monocytogenes* strain F6214-1 Tn*917* mutant bank was constructed as previously described (18). Bacteria exhibiting resistance to chloramphenicol, erythromycin, and lincomycin were screened for phage resistance by patching bacteria onto L agar plates overlaid with 3 ml of LCM soft agar containing 10^8 to 10^9 P35h4 phage per plate. Members of the mutant bank that exhibited growth under these conditions were tested further for phage resistance by standard plaquing efficiency and cross streak analysis (35).

Sequence analysis of the phage-resistant insertion mutant and cloning of the parental *glcV* gene. The Tn*917* insertion site in the phage-resistant mutant (PAS394) was initially located by arbitrarily primed PCR using previously described methods (21, 27), except that the random primer synthesis was based on the G-C composition of *L. monocytogenes*. Additional DNA sequence (i.e., sequence flanking the insertion) was obtained by using a modification of ligation-mediated PCR (36). Using this method, chromosomal DNA of strain F6214-1 was digested with a series of restriction endonucleases (BamHI, XhoI, BglII, KpnI, EcoRV, SalI, PstI, and XbaI) and ligated into pGEM digested with the same (or complementary) restriction enzymes. This resulted in eight individual ligation reaction mixtures. One microliter of each ligation mixture was PCR amplified using the pGEM forward or reverse primer (depending on the enzyme) and a primer based on the known F6214-1 sequence flanking the insertion (initially identified by arbitrarily primed PCR). The resulting amplicons from positive reactions, which were between 500 and 1,000 bp long, were sequenced. A total of 4 kb of sequence was obtained, including the sequence of an ORF presumptively containing a group 2 glycosyl transferase gene (designated *glcV*) that was interrupted by the Tn*917* insertion and an ORF encoding a putative membrane protein immediately downstream from *glcV*, designated *pmpA* (*p*utative *m*embrane *p*rotein *A*) for convenience. Information for an additional DNA sequence flanking the two genes was also obtained. This information was used to design PCR primers (5′-GC**TCTAGA**GCGAAGCTAAGACGACTCGTTCCAC and GC**TCTAGA**GCAATTTGCTTTAAAACATCCG) to clone the contiguous region containing both *glcV* and *pmpA* using the Expand high-fidelity PCR system (Roche). The resulting amplicon was digested with XbaI (sites indicated by bold type in the primer sequences) and ligated into pGEM that was digested with XbaI and treated with shrimp alkaline phosphatase. The resulting plasmids were introduced into *Escherichia coli* DH5-α by transformation, and

one clone was confirmed to have a full-length insertion. This plasmid was then digested with XbaI, and the insert was gel purified, ligated into pKSV7 digested with XbaI, and treated with shrimp alkaline phosphatase. The mixture was introduced into *E. coli* by transformation, and a resulting transformant was identified as having pKSV7 with a full-length *glcV pmpA* insert. The plasmid (pPAS29) was purified with a QIAgen midi prep kit and introduced into PAS394 (F6214-1 *glcV*::Tn*917*) by electroporation (28). The plasmid was first confirmed to restore phage sensitivity to the resistant mutant and then used to construct subclones containing either *glcV* or *pmpA* by taking advantage of conveniently located HindIII, EcoRI, and EcoRV sites. The *glcV* and *pmpA* subclones were confirmed by PCR amplification and DNA sequencing of the ligation junctions. (Sequencing was done using standard methods by McLab, South San Francisco, CA.) The in-frame *glcV* deletion was obtained by employing PCR to amplify the upstream portion of the full-length clone, including the presumptive ATG start site of *glcV*, using 5′-CCC**AAGCTT**G**TCTAGA**GGAAGCTAAGACGACTCGT TCCAC and 5′-CCC**AAGCTT**CCATTCTTAAACAACTCCTTAAAT. The product was ligated into the HindIII clone (described above), forming a 249-bp in-frame deletion, which was confirmed by sequence analysis. Following confirmation, clones were introduced into PAS394 by electroporation (see Results).

Phage plaquing efficiency and attachment analysis. Phage plaquing efficiency was determined by titrating a P35h4 lysate with the various test strains and comparing the numbers of plaques to the numbers obtained with the parental strain. Plaques were enumerated after 24 h of incubation at 22°C.

Listerial strains to be tested for phage binding were grown overnight at 31°C with shaking in 100 ml of LCM broth (chloramphenicol was added when test and control strains harbored pKSV7 or one of its derivatives). Cells were pelleted and resuspended in 0.4 ml fresh growth medium. Each suspension, containing ca. 2×10^{11} CFU/ml, was mixed with 0.4 ml of P35h4 (7×10^6 PFU/ml) in Eppendorf tubes and incubated at room temperature. At various times, 200 μl was removed, and cells were pelleted by centrifugation (4 min at 13,000 × *g*). The supernatant (containing unbound phage) was titrated with F6214-1 using standard plating procedures. The initial time point (zero time) was the time immediately after addition of the bacteria to the phage. The number of PFU obtained at zero time was defined as 100%, and the number of PFU obtained at each subsequent time point was expressed as a percentage of the initial number of PFU. A control incubation suspension identical to the suspension described above except that it lacked bacteria was employed to ensure that PFU were not eliminated from the suspension in the absence of bacteria.

Cell culture methods. Cultured mouse enterocyte (MODE K [41]) monolayers were propagated as previously described (18). Listerial strains destined for tests of binding and plaque-forming ability were grown overnight in BHI broth at 31°C with shaking. Strains harboring pKSV7 derivatives were grown in the same broth containing chloramphenicol (BHI-Cm). An overnight culture was used to inoculate 25 ml of BHI or BHI-Cm broth. The subculture was grown at 31°C until the optical density at 600 nm was 0.3 to 0.6 (logarithmic growth). An aliquot of the culture was harvested by centrifugation (7,668 × *g* for 10 min), and the cell pellet was resuspended in Dulbecco modified Eagle medium to obtain a final concentration of 1.5×10^8 CFU/ml. The efficiencies with which the various listerial strains bound and formed plaques on MODE K cells were determined as previously described (18), except that plaque formation was assessed 48 h after binding in wells fixed with 100% methanol and stained with HEMA 3 solution II (Protocol; Fisher Scientific). Plaques were enumerated and plaque sizes (area in pixels) were determined using the AlphaImager 3300v2.03 spot denso analysis tool (Alpha Innotech Corp.).

In vivo experiments. Mouse lethality was determined following oral inoculation of 20 μl of phosphate-buffered saline containing ca. 1.5×10^8 CFU of the listerial strain to be tested into groups of ca. 8-week-old female A/J mice. Percent survival was determined daily until 11 days after inoculation. Moribund mice were sacrificed and counted as dead animals.

Mouse liver and spleen infectivity indices were used to quantitate the degree to which the various listerial strains differed in their abilities to infect these organs. In these experiments, groups of three or four ca. 8-week-old female A/J mice were inoculated orally with a mixture of the parental strain (F6214-1/pKSV7) and either the uncomplemented mutant (PAS394/pKSV7) or the complemented mutant (PAS394/pPAS94) using 2×10^7 CFU of each strain (i.e., a total of 4×10^7 CFU in 20 μl). Mice were sacrificed at 48 h postinoculation, and the numbers of CFU of each strain in the spleen, liver, and colon contents were determined by plating samples onto BHI medium containing streptomycin and nalidixic acid. Sensitivity to lincomycin and erythromycin (conferred by Tn*917*) was used to distinguish the recovered strains by replica printing onto medium containing these antibiotics in addition to streptomycin and nalidixic acid (18).

Statistical and genomic methods. The standard deviation of the mean was calculated with the aid of the Microsoft Excel STDEV function and MiniTab14. The standard error was calculated by dividing the standard deviation by the square root of the number of samples. The statistical significance of mean differences was determined using Student's *t* test with the aid of the Microsoft Excel TTEST function. The probability of error threshold was a P value of <0.05.

The DNA sequence was analyzed for ORFs using orf finder at NCBI and WinGene. Amino acid sequences were used to compare proteins to known proteins in NCBI databases using protein BLAST. Genes flanking the *glcV* and *pmpA* genes in *L. monocytogenes* serotype 4b, *L. innocua*, and *L. welshimeri* were identified using NCBI Entrez Genome. A similar region in *L. monocytogenes* EGD-e was identified using the flanking genes (*flgJ* and *galU*) at NCBI Entrez Genome and *ListList* (Institute Pasteur).

Nucleotide sequence accession number. The entire sequenced *glcV* region of *L. monocytogenes* strain F6214-1 has been deposited in the GenBank database under accession number EU499188.

RESULTS

Isolation and initial characterization of the bacteriophage-resistant mutant. The phage-resistant mutant chosen for this study was identified by patching individual members of our Tn*917* insertion bank onto petri plates with agar overlays containing ca. 10^9 PFU of P35h4. Candidate resistant isolates were recovered from the bank and colony purified. By screening our bank rather than by employing a selective procedure, the phage-resistant mutant was identified without exposure of the catalogned bank to phage. Phage resistance was confirmed in a variety of tests, including phage plaquing efficiency, as well as cross streaking. A single isolate that did not support plaque formation at any multiplicity of infection tested was chosen for further study and designated strain PAS394.

PAS394 was attenuated in mice inoculated orally, as indicated by time-to-death studies, and was defective in the ability to form normal-size plaques on cultured mouse enterocytes (Fig. 1A and 1B). These properties indicated that a more thorough examination of the mutant was warranted.

Location and genomic setting of the insertion in the phage-resistant mutant. The insertion site associated with phage resistance in PAS394 was located using arbitrarily primed PCR, and a 4-kb region (flanking the insertion site) was subsequently sequenced using ligation-mediated PCR. The insertion mapped to the 3′ end of a provisional gene (which we designated *glcV*) predicted to encode a group 2 glycosyl transferase. Genes similar to *glcV* are found in a relatively conserved region of the chromosomes of the fully sequenced listeriae, including *L. monocytogenes* serotype 4b (accession number NC_002973) (25), *L. innocua* serotype 6a (NC_003212) (42), and *L. welshimeri* serotype 6b (NC_008555) (17) (Fig. 2). The conserved region extended to an adjacent ORF predicted to encode a hypothetical membrane protein. (The properties of the latter ORF were not studied, but the ORF was cloned initially along with *glcV* and designated *pmpA* for convenience.) The F6214-1 chromosomal organization in the *glcV-pmpA* vicinity was most similar to the chromosomal organization of *L. welshimeri*, with the *flgJ* gene (encoding a provisional *N*-acetylmuramoyl-L-alanine amidase [family 4]) immediately upstream from these genes and the *galU* gene (encoding a provisional UTP-glucose-1-phosphate uridylyltransferase) immediately downstream (Fig. 2). The *L. monocytogenes* F6214-1 *glcV* gene showed strong similarity (97% identity and 98% positive) to an *L. welshimeri* serotype 6b strain SLCC5334 gene and moderate similarity to an *L. monocytogenes* serotype 4b strain F2365

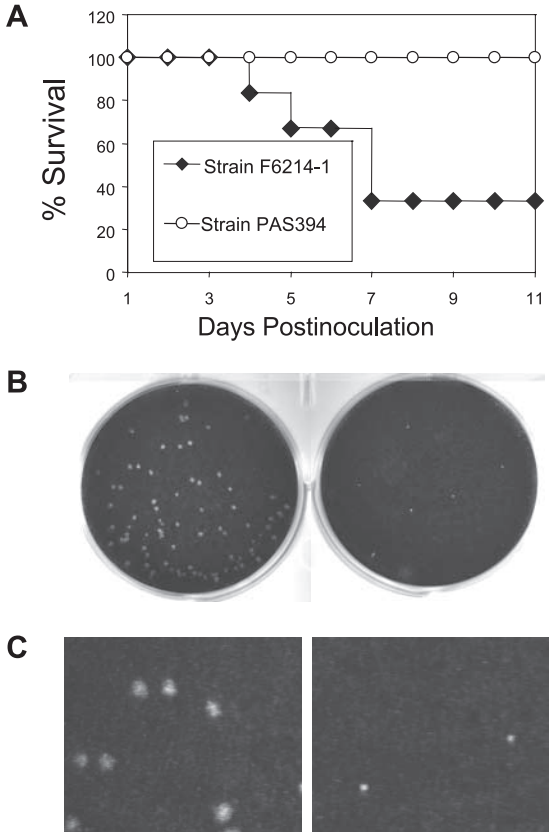

FIG. 1. (A) Lethality of the parent F6214-1 strain compared to phage-resistant mutant PAS394 in mice following oral inoculation of ca. 1.5×10^8 CFU. Six mice received F6214-1, and eight mice received PAS394. Percent survival was determined by dividing the number of mice that lived by the total number of mice inoculated. (B) Plaques on a mouse enterocyte (MODE K) monolayer 48 h after inoculation with equivalent numbers of CFU of the parent (F6214-1) (left well) and the phage-resistant mutant (PAS394) (right well). (C) Plaques of F6214-1 (left panel) and PAS394 (right panel) at a magnification of ×500. MODE K plaquing experiments were performed as described in the text. Representative wells from sets of experiments performed in triplicate are shown.

gene (76% identity and 89% positive) and an *L. innocua* serotype 6a strain Clip11262 gene (77% identity and 89% positive). Most strikingly, the F6214-1 *glcV* product showed no significant similarity to the product of any glycosyl transferase gene in *L. monocytogenes* serotype 1/2a strain EGD-e. A comparison of the genomic region revealed the presence of *flgJ* (lmo101076), *orfY* (lmo1077), and *galU* (lmo1078) (Fig. 2). No genes similar to *glcV* or *pmpA* are in this area of the EGD-e genome.

Genetic complementation in *trans* restored phage sensitivity. Complementation experiments were conducted to determine if the disrupted *glcV* gene in PAS394 was responsible for phage resistance. PCR was used to facilitate cloning of the *glcV-pmpA* region into pKSV7. Modifications of this construct were made using conveniently located HindIII, EcoRI, and EcoRV sites (Fig. 3A).

Constructs containing an intact *glcV* gene complemented the chromosomal insertion mutation of strain PAS394, restoring sensitivity to phage P35h4 (as shown by cross streak tests [Fig. 3B]). The mutant (PAS394) containing plasmids that lacked the 3′ end of *glcV* or contained an in-frame deletion of *glcV*, eliminating 28% of the coding region, was phage resistant. The presence of an intact *pmpA* gene was irrelevant for restoration of phage sensitivity. We concluded from these results that *glcV* produced a product that was necessary and sufficient to restore phage sensitivity to the mutant.

Plaquing efficiency tests provided a sensitive quantitative corollary to the qualitative cross streak tests by showing that *glcV* was key to restoring phage sensitivity (Fig. 4). With one exception (pPAS64), all constructs showing complementation by the cross streak test increased plaquing efficiency from zero (no plaques) to a level indistinguishable from that of the parent (ca. 5×10^9 PFU). It was unclear why pPAS64 did not complement as fully as the other constructs bearing the parental *glcV* gene. However, while statistically noteworthy, the difference was decidedly minor. We chose pPAS94 as the plasmid to employ in subsequent complementation tests because it was the smallest of the *glcV*-containing clones created and it restored phage sensitivity to a level indistinguishable from that of the parent (Fig. 3B and 4). Below, we compare the complemented mutant (PAS394/pPAS94 [phage sensitive]) with the uncomplemented mutant (PAS394/PKSV7 [phage resistant]) and the parental strain harboring the cloning vector (F6214-1/pKSV7 [phage sensitive]).

Phage binding by the mutant and effect of complementation. We suspected that the lack of a functional *glcV* product would alter the surface of PAS394 and prevent phage attachment. We tested this idea by employing a phage binding assay. The results of this assay, in which bacteria were used to absorb and thus titrate phage over time, clearly showed (Fig. 5) that the resistant mutant harboring the cloning vector (PAS394/pKSV7) bound negligible numbers of phage. In contrast, the phage binding ability of the complemented mutant (PAS394/pPAS94) was indistinguishable from that of the parent.

Mouse enterocyte (MODE K) plaquing by the mutant and effect of complementation. MODE K binding and plaquing analysis revealed that the parent, mutant, and complemented mutant were indistinguishable in terms of enterocyte binding efficiency (number of bacteria bound/number of bacteria added) (Fig. 6A). However, the phage-resistant mutant had a dramatically lower plaquing efficiency (number of plaques formed/number of bacteria bound) than the parental control. The plaquing efficiency of the complemented strain was indistinguishable from that of the parent (Fig. 6B). In addition to the lower plaquing efficiency exhibited by the resistant mutant, the plaques that were produced were minute compared to those observed for the parent (Fig. 6C). Complementation resulted in a plaque size indistinguishable from that observed for the parent, as determined quantitatively (Fig. 6C) and qualitatively (Fig. 6D).

Oral infectivity of the mutant and effect of complementation. The reduced plaquing efficiency and plaque size of the mutant on MODE K cells were reflected in the results of competitive index infectivity experiments in vivo. In these experiments, mice were orally inoculated with a mixture containing approximately equal numbers of the parent and the complemented or uncomplemented mutant. Mice were sacrificed at 48 h postinoculation, and the numbers of CFU of each strain in the liver, spleen, and colon contents were determined. Vir-

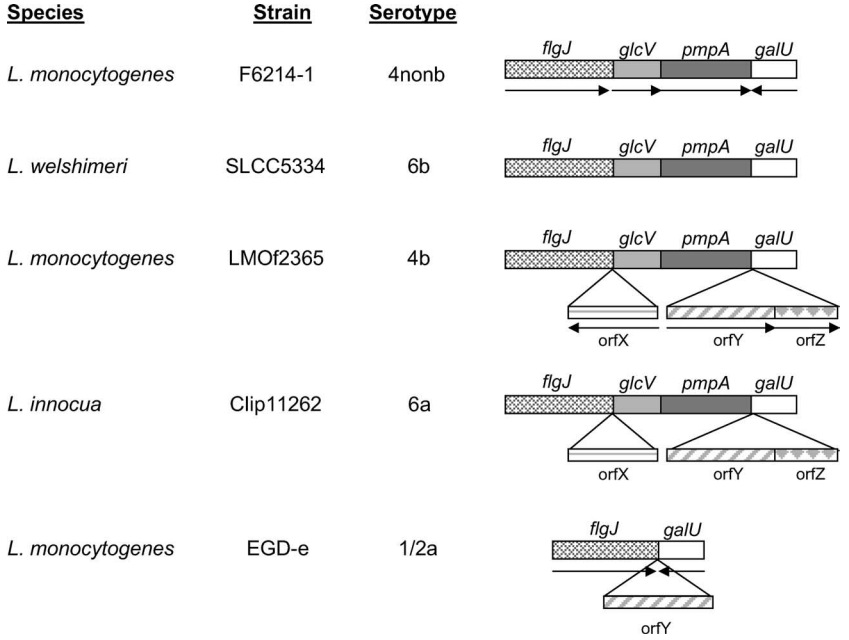

FIG. 2. Genomic alignment of the group 2 glycosyl transferase gene (*glcV*) in F6214-1 and other listeriae. Sequences of fully sequenced species and serotypes of *Listeria* are shown in decreasing order of *glcV* similarity. *L. monocytogenes* EGD-e has no ORF similar to *glcV*. The same pattern indicates strong identity across the strains shown. ORFs are contiguous within each strain. Specific *glcV* identity levels are indicated in the text. The genomic designations (Entrez Genome) are as follows: *L. welshimeri*, lwe1056 to lwe1059; *L. monocytogenes* serotype 4b, lmof23651093 to lmof23651099; *L. innocua*, lin1064 to lin1070; and *L. monocytogenes* EGD-e, lmo1076 to lmo1077. The sequenced region of strain F6214-1 has been deposited in the GenBank database under accession number EU499188.

ulence was measured by determining the colonization levels in the spleens and livers of individual mice. The degree of attenuation exhibited by the phage-resistant mutant was very dramatic (no uncomplemented mutant CFU were recovered from any liver or spleen in any mouse examined). Complementation resulted in a level of infectivity that was indistinguishable from that of the parental control (Fig. 7). The colon contents of inoculated mice included strains of both mutant and complemented mutant (parental) phenotypes at indistinguishably different levels. However, identification of the parental strain (i.e., the internal control) was sometimes complicated by the presence of resistant enterococci. This precluded colonic measurements that were the same quality as those for the other organs.

DISCUSSION

Here we describe attenuation of *L. monocytogenes* strain F6214-1 produced by the loss of the *trans*-acting product of a gene, provisionally designated *glcV*, that is also necessary for phage attachment. This gene is predicted to encode group 2 glycosyl transferase activity and is found in several of the most well-described *Listeria* species.

Phage resistance was employed as a strategy to identify attenuated mutants because such relationships have been described in the past (11, 31, 35, 39). Whereas this strategy has not been specifically applied to listeriae, Autret et al. (2), using signature-tagged mutagenesis, identified a gene (*gtcA*) whose putative product is required for sensitivity of a serotype 1/2a strain to phage LMUP121 (19). Mutants with insertions in *gtcA* were isolated as organisms that were unable to cross the blood-brain barrier after parenteral inoculation of mice with strain EGD-e. The *gtcA* product is suspected to effect the decoration of teichoic acid with rhamnose in serotype 1/2 strains and effects the decoration by *N*-acetylglucosamine in the serotype 4 strains (40). The gene that we describe here, *glcV*, shares no identity with *gtcA* from either the serotype 1/2a or serotype 4b strains. Nevertheless, it does encode the same inferred activity (glycosyl transferase). It seems clear that the association of attenuation with phage resistance is not limited to our strain of *L. monocytogenes* and could be a feature of particular classes of listerial phage-resistant mutants (8, 10, 40).

We took the precaution of isolating our phage-resistant mutant by screening rather than selection so that the mutant bank would not be subject to artifactual effects, such as lysogeny or pseudolysogeny (20). The phage employed in the isolation of our resistant strain has not been documented to be lysogenic (19). However, analyses of the listeriae for which genomic sequences are available have revealed a number of cryptic phage (25). Consequently, phage infection and recombination could produce survivors with unexpected properties. To avoid these unwanted artifacts, we employed screening for resistance rather than selection for resistance. The choice of the P35h4 phage was not predicated on knowledge of its particular biology or binding properties. Within its normal host range, the parental phage (P35) likely recognizes features of the cell surface that require the presence of rhamnose as a decoration on serotype 1/2 teichoic acid (2, 40). However, the actual receptors for P35 and its derivative P35h4 (employed in this study) have not been identified.

The lesion conferring phage resistance mapped to an ORF

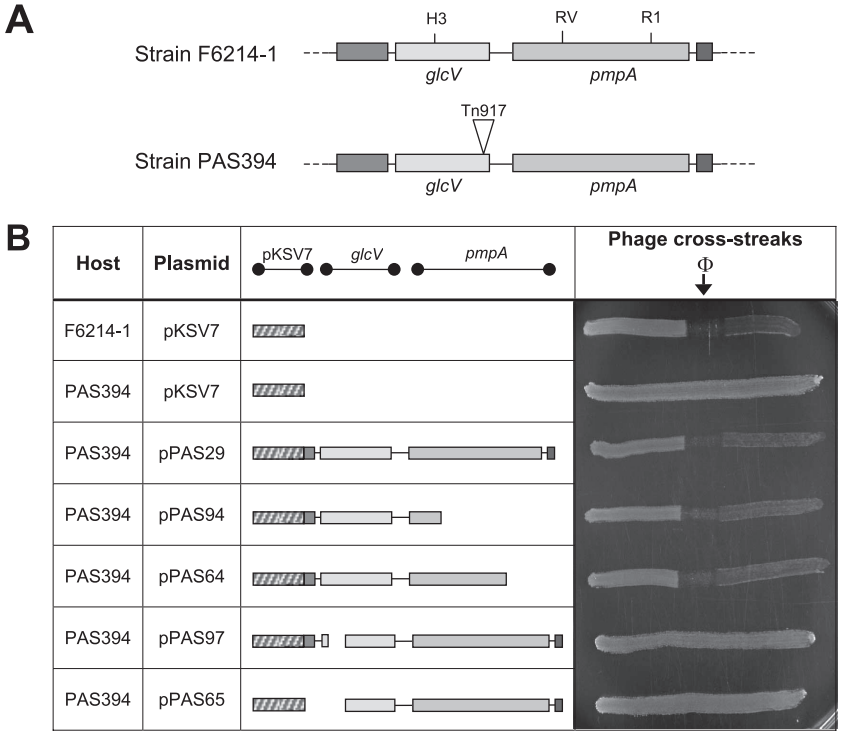

FIG. 3. (A) Location of the lesion defining *glcV* and (B) recovery of the phage sensitivity phenotype in the *glcV* mutant (strain PAS394) by *glcV* complementation in *trans*. Phage sensitivity or resistance was determined by a cross streak test employing phage P35h4 struck vertically. Individual host-plasmid combinations were struck horizontally. Growth to the right of the vertical phage streak indicates resistance, and no growth indicates sensitivity to the phage. Restriction endonuclease sites are designated as follows: H3, HindIII; R1, EcoRI; and RV, EcoRV. The F6214-1 parental allele sequence has been deposited in the GenBank database under accession number EU499188. The Tn*917* insertion site in PAS394 is at nucleotide position 1623 of the parental sequence.

that we provisionally designated *glcV*. The gene produced a product that is presently assumed (based on sequence comparisons) to have group 2 glycosyl transferase activity (based on annotation of the accession number NC_002973 sequence [25]). The product of the *glcV* gene in strain F6214-1 has the highest level of amino acid sequence similarity to the product of *glcV* in *L. welshimeri* (a serotype 6 nonpathogenic species), followed by the product of the gene in *L. monocytogenes* serotype 4b. Interestingly, our *glcV* shows essentially no similarity to any gene encoding a group 2 glycosyl transferase in *L. monocytogenes* serotype 1/2. One way to account for this absence is to assume that the *glcV* gene product recognizes sim-

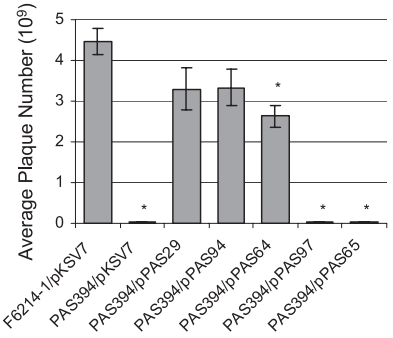

FIG. 4. Plaquing efficiency of phage P35h4 for the host-plasmid combinations indicated on the *x* axis. F6214-1 was the parental strain used, and PAS394 was the phage-resistant mutant used. The plasmids harbored by the strains are as follows: pKSV7 (vector), pPAS29 (pKSV7 *glcV pmpA*), pPAS94 (pKSV7 *glcV* Δ*pmpA1*), pPAS64 (pKSV7 *glcV* Δ*pmpA2*), pPAS97 (pKSV7 Δ*glcV1 pmpA*), and pPAS65 (pKSV7 Δ*glcV2 pmpA*). Asterisks indicate values that are significantly different ($P < 0.05$) from the value for the parental host-plasmid combination (F6214-1/pKSV7).

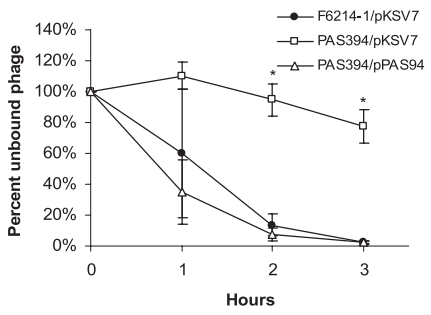

FIG. 5. Titration of phage P35h4 by the host-plasmid combinations indicated. The host-plasmid combinations were mixed with phage as described in the text, and bacteria were removed by centrifugation at the times indicated. Relative phage binding ability is expressed as a percentage of phage present at the time of addition of bacteria (zero time). F6214-1 was the parental strain used, and PAS394 was the phage-resistant mutant used. The pKSV7 plasmid was the vector, and pPAS94 was the vector containing the parental *glcV* gene. Asterisks indicate values that are significantly different ($P < 0.05$) from the value for the parental host-plasmid combination (F6214-1/pKSV7).

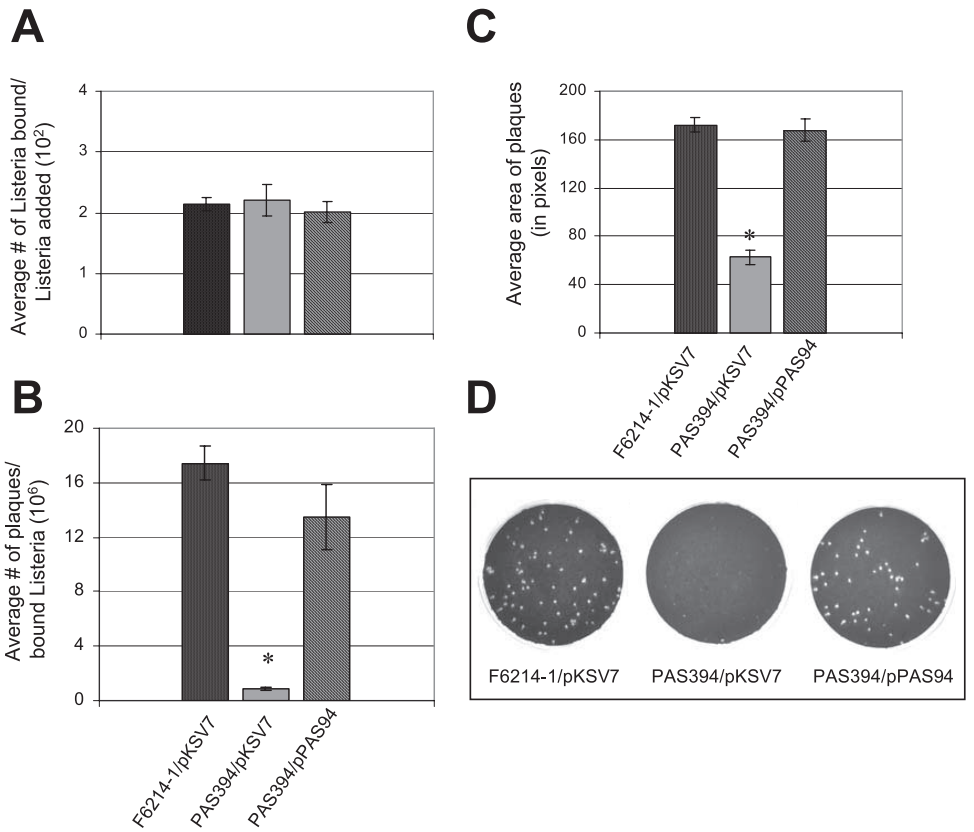

FIG. 6. Listerial binding and plaquing ability on mouse enterocytes (MODE K cells). The plaquing abilities of the mutant (PAS394/pKSV7) and the complemented mutant (PAS394/pPAS94) were compared to that of the parent (F6214-1/pKSV7). (A) Initial binding, number of listeriae added to MODE K monolayer wells that remained attached after incubation for 10 min, and removal of unbound bacteria. (B) Number of plaques formed per number of bacteria bound. (C) Average plaque size (plaque area expressed in pixels). Each graph represents data from three separate experiments performed in triplicate. The error bars indicate standard errors. Asterisks indicate values that are significantly different ($P < 0.05$) from the value for the parent (F6214-1/pKSV7). (D) Plaques on a mouse enterocyte (MODE K) monolayer 48 h after inoculation with equivalent numbers of CFU. Plaquing experiments were performed as described in the text. Representative wells from sets of experiments performed in triplicate are shown.

ilar features in the teichoic acids of serotype 6 and 4 strains that are absent in the serotype 1/2 strains (30). Additional studies are required to definitively establish that *glcV* encodes a product with enzymatic activity and to identify its natural substrate.

We employed complementation in *trans* to prove that the *glcV* gene produced a product that conferred phage sensitivity. The smallest clone employed contained a single ORF (i.e., no other genelike sequences were detected in the clone when it was examined by using WinGene 2.31). Concerns that the Tn*917* insertion defining *glcV* could be polar, while historically well founded, were not supported by our observations.

In our phage attachment assay, the mutant harboring just the cloning vector absorbed an insignificant number of phage particles over the course of the experiment, whereas the complemented strain and the parent displayed absorption profiles that were indistinguishable from each other. We inferred from this experiment that a cell surface defect is sufficient to confer phage resistance.

Mutants, complemented and uncomplemented, were examined for their ability to bind to cultured mouse enterocyte (MODE K) monolayers, the efficiency with which plaques were formed, and the size of the plaques produced. The mutant exhibited a MODE K binding efficiency (number of bacteria bound/number of bacteria added) indistinguishable from that of the parent or the complemented mutant. However, the efficiency with which the bound bacteria went on to form visible plaques (number of plaques formed/number of bacteria bound) was severely affected by the mutation (the mutant displayed an efficiency that was approximately 1/10 that of the parent or the complemented mutant). In addition to the lower plaquing efficiency, the average mutant plaque size differed significantly from the average parent plaque size (the plaque size was restored to parental dimensions by *glcV* supplied in *trans*). Monolayers were not examined microscopically for plaques. Thus, it remains possible that the mutant produced a combination of observed and microscopic plaques with an efficiency similar to that of the parent. We did confirm that the mutant plaques seen were unlikely the result of intra- or extragenic reversion events by reinfecting monolayers with progeny from several plaques and confirming that the plaque size and plaquing efficiency were similar to those of the original mutant.

The retention of enterocyte binding ability by the phage-

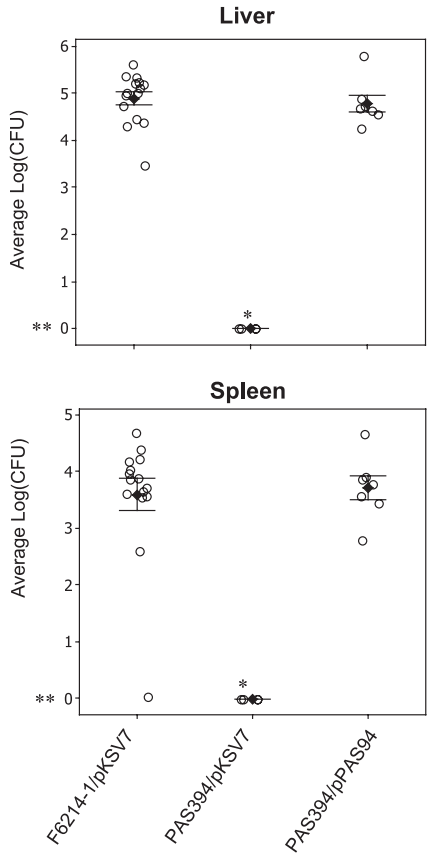

FIG. 7. Numbers of CFU of strains indicated on the *x* axis from the livers and spleens of individual mice treated as described in the text. The average number of CFU per organ is indicated by a filled diamond, and the standard error of the mean is indicated by the error bars. The results of two separate experiments, each performed with three or four mice per group, are shown. The strain designations are as follows: F6214-1/pKSV7 (parental strain harboring the pKSV7 cloning vector), PAS394/pKSV7 (mutant harboring the pKSV7 vector), and PAS394/pPAS94 (mutant harboring the parental *glcV* allele cloned into the pKSV7 vector). Single asterisks indicate values that are significantly different ($P < 0.05$) from the value for the parent. Two asterisks indicate that the zero indicated on the *y* axis refers to the actual number of CFU recovered.

resistant mutant implied that the defect leading to lower plaquing efficiency and plaque size occurred after binding. This finding was somewhat unusual. We and other workers have found that bacterial cell surface alterations that prevent phage attachment have a measurable effect on host cell binding efficiency (11, 31, 35, 39). It could be that the interaction of the *glcV* mutant with enterocytes is indeed fundamentally altered but that this was not reflected by a measurable change in avidity. Another alternative is that the absence of the *glcV* product has an unappreciated effect in listerial intermediary metabolism that leads to an intracellular growth defect (9). However, our *glcV* mutant exhibited no demonstrable deviation from the parent in terms of its growth properties in the laboratory, and it is well documented that alterations in bacterial surface features can influence intracellular growth (5, 43). Inspection of mutant colonies on standard indicator medium revealed that the *glcV* lesion had no noticeable effect on hemolysin production, phospholipase production, or motility. However, indirect effects of the lesion on previously described virulence factors remain a possibility. Efforts to better define the reasons for the small-plaque phenotype exhibited by the mutant are under way.

The in vivo infectivity of the phage-resistant mutant was measured by performing competitive index experiments. This method was chosen for its well-known precision in distinguishing between parent and mutant strains (1). Given our results, the precision was not needed because in all instances examined, no mutants were recovered from the liver or spleen. In contrast, the parental strain and the complemented mutant were both recovered at the same high levels in most of the organs sampled. Clearly, phage resistance resulted in attenuation that was completely reversed by complementation.

Our discovery of a listerial mutant that is avirulent via the oral route in mice adds to the relatively short list of factors that are required for listerial pathogenesis via the natural route of infection (13, 32). We anticipate that further study of orally virulent *L. monocytogenes* will lead to improvements in identifying the full range of lesions that affect all infective steps in the host.

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